

Effects of forest patch size on physiological stress and immunocompetence in an area-sensitive passerine, the Eurasian treecreeper (*Certhia familiaris*): an experiment

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We manipulated the primary brood size of Eurasian treecreepers (*Certhia familiaris*) breeding in different sized forest patches (0.5–12.8 ha) in moderately fragmented landscapes. We examined the effects of brood size manipulation (reduced, control, enlarged) and forest patch size on physiological stress (heterophil–lymphocyte ratios; H/L), body condition and cell-mediated immunocompetence (phytohaemagglutinin test). Nestlings' H/L ratios were negatively related to forest patch area in control and enlarged broods, whereas no effects were found in reduced broods. The effects of forest patch area were strongest in enlarged broods, which had, in general, twofold higher H/L ratios than control and reduced broods. The elevated H/L ratios were positively related to nestling mortality and negatively correlated with body-condition indices suggesting that the origin of stress in nestlings was mainly nutritional. Cell-mediated immunity of nestlings was not related to brood manipulation or to forest patch size. Also, the H/L ratios of adults were not related to brood manipulation or forest patch size. In addition, parental H/L ratios and body condition were not related to nestling H/L ratios. Our results suggest that during the breeding period the deleterious effects of habitat loss are seen explicitly in growing young.

Keywords: physiological stress; heterophil–lymphocyte ratio; H/L ratio; area sensitivity; habitat loss; treecreeper

1. INTRODUCTION

The survival of populations may show a threshold response to habitat loss, considered the most important factor causing the current species extinction event (Groombridge 1992; Bibby 1995; Thomas & Morris 1995; Fahrig 1999). In forest landscapes, the first results of habitat loss are decreases in forest patch size and increases in patch isolation (Fahrig 1999). Adverse effects of decreasing patch sizes are explicitly found for interior species (Bender *et al.* 1998). Investigations into the causes of area sensitivity have focused primarily on nest predators and brood parasites (reviewed in Paton 1994). Additionally, area sensitivity can be caused by chronic food shortage in small forest fragments (Zanette *et al.* 2000). Patch size may influence the nutritional status of growing nestlings, because adults feeding altricial young will be constrained in their foraging by the location of the nest (Hinsley *et al.* 1999). Lack of food may be related to chronic physiological stress in individuals (Romero & Wikelski 2001; Suorsa *et al.* 2003). Long-term (i.e. days or weeks) physiological stress, in turn, may depress resistance to diseases by depleting lymphoid cells and

leucocytes (Harvey *et al.* 1984; Morici *et al.* 1997), prolong the mobilization of stored energetic resources and render the animal susceptible to fatigue (reviewed in Buchanan 2000). Thus, stresses that occur early in the life of the growing bird may have a long-lasting impact on reproductive performance and survival (Lindström 1999).

Nutritional stress in nestlings may be detected indirectly from growth rates, but these inevitably incorporate genetic and maternal effects not necessarily related to nutritional condition during the growing period (Merilä 1996). A more direct way of estimating nutritional stress and immunocompetence is through measurements derived from individual peripheral blood samples (Moreno *et al.* 2002). The heterophil–lymphocyte ratio (H/L) has proved sensitive to increased plasma corticosterone levels (Morici *et al.* 1997; Post *et al.* 2003) and a reliable physiological indicator of stress in wild bird populations (Hörak *et al.* 1998; Ots *et al.* 1998). Even in growing chicks, still possessing an incomplete immune system, high H/L ratios have been found to impair growth rates (Moreno *et al.* 2002).

Poor nutrition is also known to be associated with lowered immunocompetence, i.e. the ability of the immune system to react to antigen challenge (Ilmonen *et al.* 2003). Further, high within-brood competition for resources in experimentally enlarged broods is related to suppressed immunocompetence (Saino *et al.* 1997; Hörak *et al.* 1999; Ilmonen *et al.* 2003). Immunocompetence is routinely

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measured by way of cell-mediated immunity by using injection with a novel mitogen (phytohaemagglutinin; PHA), because it is easily applied in the field and does not provoke potential confounding effects associated with physiological stress (Merino *et al.* 1999). The method is considered a useful tool for evaluating thymus-dependent immune function (Goto *et al.* 1978).

There is empirical evidence for adverse effects of decreasing woodland area on breeding parameters of passerines under conditions of extreme woodland fragmentation (Hinsley *et al.* 1999; Zanette *et al.* 2000). However, there is a lack of experimental data on area sensitivity and research carried out within landscapes subject to only moderate levels of forest fragmentation. We conducted a brood size manipulation experiment in forest patches of different sizes to examine the area effects on physiological stress and immunocompetence in the Eurasian treecreeper (*Certhia familiaris*), an area-sensitive passerine preferring forests over 100 years old (Haila *et al.* 1989; Virkkala *et al.* 1994; Väisänen *et al.* 1998). The first individual birds start breeding in scant food conditions in early April when snow cover and night frost still prevail in central Finland. The species strictly avoids open habitats and specializes in searching for invertebrates, especially spiders (65% of the nestling food mass) on large tree trunks (Suhonen & Kuittunen 1991). As a result of adaptation to forested areas, and because treecreepers readily accept nest-boxes as breeding sites, it is an ideal species for gathering data on area sensitivity in forest-dwelling birds.

Our goals were to establish:

- (i) whether the H/L ratios of manipulated and control broods and of their parents vary as a function of forest patch area,
- (ii) whether the T-lymphocyte responses of manipulated and control broods vary with forest patch area,
- (iii) whether the H/L ratios are related to the growth rate, T-cell response and survival in nestlings,
- (iv) whether the body condition and the H/L ratios of parents are associated with the H/L ratios of the broods.

2. MATERIAL AND METHODS

(a) Study area

H/L ratios and T-cell response data were collected from the study area covering 1150 km² in central Finland (centred on 62°37' N, 26°20' E) in the summer of 2001. Forested land covered 71% of the study area; the remaining 29% consisted of open habitats, such as cultivated fields, clear-cuts, lakes, treeless bogs and other open habitats. The most common forest types are coniferous forests dominated by Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), which cover 42% and 21% of the forested land, respectively. Deciduous forests and mixed forests of conifers, birches (*Betula* spp.) and other deciduous trees cover 30% of the forested area. The remaining 7% consists of saplings and spruce and pine mires.

Two nest-boxes were placed 30 m apart in the interiors of 229 forest patches in the autumn of 1998. Each forest patch contained two nest-boxes to allow for the possibility of a second breeding attempt by the territorial pair. Only one treecreeper pair occupied a nest-box pair at a time; thus we regarded the

midpoint of the nest-box pair as a territory centre and used territory as a sampling unit. Out of the 229 nest-box pairs, 99 were occupied (minimum of one egg laid) and considered territories in the spring of 2001. Out of these 99 territories, 63 were used in the brood size manipulation experiment.

(b) Brood size manipulations

Based on direct observations in the field, provisioning trips of treecreeper parents extend to a distance of 200 m from the nest (H. Hakkarainen, unpublished data). Hence, the forested area (ha) around a nest-box was established to a radius of 200 m (covering maximum of 13 ha). We used classified Landsat TM 5 satellite images (from 1995 to 1997) produced by the National Land Survey of Finland (NLS) (Vuorela 1997). The satellite-image-based land-use and forest data were imported to a Geographic Information System (GIS). The forested area around the nest-box was defined by summing the total area of pixels containing at least 50 m³ ha⁻¹ of timber. By using this criterion, a forest patch was clearly separable from the neighbouring open habitats and saplings in the field. Reductions in the area of forest patches as a result of forest clear-cuts made after 1997 were updated in the field. The size of occupied forest patches ($n = 99$) varied from 0.5 to 12.75 ha (mean \pm s.d. = 7.38 \pm 2.89 ha).

The mean area of occupied forest patches was used in defining small (less than 7.38 ha) and large (greater than 7.38 ha) forest patches. We then formed triplets within both small ($n = 9$) and large ($n = 12$) forest patches consisting of three synchronously hatched (± 1 day) nests. The proportions of the main stem species (spruce, pine, birch, other deciduous stems) measured within the forest patches ($n = 63$) (see details in Suorsa *et al.* 2003) were not related to the forest patch size (Pearson $|r| < 0.201$, $p > 0.121$ in all of the comparisons). A triplet involved reduced (-1 chick), control and enlarged ($+1$ chick) broods. We swapped one randomly selected (2 ± 1 days) nestling from the 'reduced' nest to the 'enlarged' nest; the original clutch size was not altered in the control nests. The clutch sizes before manipulation were 5.1 ± 0.2 (mean \pm s.e.) in enlarged broods, 5.45 ± 0.1 in control broods and 5.1 ± 0.1 in reduced broods (ANOVA: $F_{2,59} = 2.446$, $p = 0.0954$). After manipulation the brood sizes differed among the three groups, being 6.1 ± 0.2 , 5.45 ± 0.1 and 4.1 ± 0.1 , respectively ($F_{2,59} = 62.682$, $p < 0.0001$). The treatment and control nests within a triplet were selected randomly. Because the parents may adjust the clutch size in relation to habitat quality (Högstedt 1980), we replaced the unhatched eggs with nestlings ($n = 6$) originating from synchronously hatched nests not included in the experiment. All work was carried out with the first broods.

(c) H/L ratio, haematocrit, biometry and T-lymphocyte responsiveness of chicks and adults

In the majority of avian species, lymphocytes are the most numerous, and heterophils the second most numerous, types of leucocytes in blood circulation (Maxwell & Robertson 1998). Heterophils are bactericidal phagocytosing cells that enter tissues during the inflammatory response (Maxwell & Robertson 1998). In contrast to the highly specific response of lymphocytes, the response of heterophils is non-specific (Jurd 1994). The H/L ratio increases in moderately stressful conditions and consequently it can be used to detect the presence of physiological stress for most stressors (Maxwell & Robertson 1998).

The H/L ratio was determined after Ilmonen (2001). A drop of blood from 11-day-old nestlings was smeared on individually marked microscope slides, air dried, fixed in absolute ethanol and later stained with conventional Romanowsky–Giemsa dye. The proportions of different types of leucocytes were assessed by examining a total of 100 leucocytes under magnification, $\times 1000$ with oil immersion. Blood samples of $43 \pm 9 \mu\text{l}$ (mean \pm s.d.) were taken in heparinized microhaematocrit capillary tubes and centrifuged at 835.146g for 10 min. We measured the thicknesses of the erythrocyte-containing layer and the plasma-containing layer to the nearest 0.01 mm using a sliding calliper and calculated a haematocrit value, the relative amount of red blood cells in the total blood volume. In adults (caught by mist-netting), the smears were collected when the chicks were 11 days old. At the same time, we also measured individual body mass to the nearest 0.1 g, maximum wing length to the nearest 0.1 mm (Svensson 1992) and subcutaneous fat index (Kaiser 1993) and condition of pectoral muscle (Gosler 1991) on five-point scales (0–4) both in chicks and in parents.

We evaluated the T-cell-mediated immunity (CMI) in 11-day-old treecreeper young, because the wing webs (anterior patagia) were then large enough to enable us to carry out the test. We injected the right wing web intradermally with 0.15 mg of PHA (Sigma, L-8754) in 30 μl of phosphate-buffered saline. PHA is a plant-derived lectin from the red kidney bean (*Phaseolus vulgaris*), which has a strong mitogenic effect on T lymphocytes. Administration of PHA induces macrophage infiltration and a dense perivascular accumulation of T lymphocytes (e.g. Goto *et al.* 1978; McCorkle *et al.* 1980). We measured the thickness of the wing web with a digital spessimeter with a constant pressure (Mitutoyo, Japan, 573-191-10) to the nearest 0.01 mm three times prior to, and 24 h after, injection and the difference in wing-web swelling was used as a measure of CMI (Smits *et al.* 1999). A control injection of phosphate buffered saline in the left wing, to control for any swelling as a result of the injection, was not done as this has previously been proved to be unnecessary (Smits *et al.* 1999). Finally, all the nests were checked after the chicks had fledged to determine the nestling mortality. One of the experimental 'enlarged' nests was predated and consequently excluded from the analyses; thus nest predation did not confound our results.

(d) Statistical analyses

We used generalized linear models with a normal error term to model the variations in H/L ratios and CMI (dependent variables) in relation to the brood size manipulation (factor), forest patch size (covariate) and their two-way interaction. Logistic regression with a binary error term was used to model the H/L ratios (covariate) and nestling mortality (proportion: [number of deceased nestlings/brood size] as a dependent variable). These analyses were carried out in proc GENMOD (SAS 8.2). Tests for adults were conducted separately for females ($n = 55$) and males ($n = 54$).

3. RESULTS

The H/L ratios of 11-day-old chicks were negatively related to the forest patch size suggesting that the physiological stress of nestlings was elevated in small forest patches (table 1; figure 1). The effect of forest patch area was not parallel in the treatment and control broods (see significant interaction term in table 1). The adverse effects of forest patch size on H/L ratios were explicitly found in

the enlarged and control broods, whereas the forest patch size did not affect H/L ratios in the reduced broods (figure 1). In general, the H/L ratios of enlarged broods were higher (0.93 ± 0.17 ; mean \pm s.e.) than those of the control (0.57 ± 0.04) and reduced broods (0.44 ± 0.05) (table 1). The size of forest patch area was positively associated with the nestling body mass ($\chi^2 = 7.40$, d.f. = 1, $p = 0.0065$), the amount of subcutaneous fat ($\chi^2 = 9.04$, d.f. = 1, $p = 0.0026$) and the condition of the pectoral muscle ($\chi^2 = 9.69$, d.f. = 1, $p = 0.0019$). The effects on body mass and pectoral-muscle condition were parallel across brood size manipulation groups (interactions, $p > 0.5346$), but there was a marginally non-significant interaction between brood size manipulation group and the amount of subcutaneous fat ($\chi^2 = 5.58$, d.f. = 2, $p = 0.0616$).

Mean H/L ratios of the broods were positively associated with the within-brood mortality rate (logistic regression GENMOD: $\chi^2 = 17.39$, d.f. = 1, $p < 0.0001$) and the effect of H/L ratios on chick mortality did not vary across brood size manipulation groups (interaction term, $p = 0.1271$). In addition, the growth rate and body condition parameters were negatively correlated with the H/L ratios further supporting the idea that H/L ratios are a good indicator of physiological stress in treecreeper chicks (table 2). CMI as measured by the PHA test was not related to the forest patch size or to the brood size manipulation. It was weakly correlated with wing length and body mass but not with the amount of subcutaneous fat, pectoral muscle condition, haematocrit or H/L ratios (table 2). The nestling mortality did not differ between the experimental (enlarged and reduced combined; $23.8 \pm 4.9\%$) and control ($18.5 \pm 7.1\%$) broods (ANOVA: $F_{1,59} = 0.389$, $p = 0.535$).

In adult males and females, the brood size manipulation and the size of forest patch did not affect their H/L ratios (GENMOD $p > 0.1167$; interaction term, forest patch \times brood size manipulation: $p > 0.2065$ in all of the comparisons). Additionally, neither the H/L ratio nor the body condition of parent birds (mass, condition of pectoral muscle, subcutaneous fat) were correlated with nestling H/L ratios or nestling mortality (Pearson $r < 0.216$, $p > 0.1164$ in all of the comparisons). The experiment did not affect adult survival and no nests were deserted as a result of our work.

4. DISCUSSION

Our results demonstrate that treecreeper nestlings growing in the small forest patches experienced elevated peripheral blood H/L ratios compared with the nestlings growing in the large forest patches. The enlarged broods had significantly higher H/L ratios than the control and reduced broods. In support of this, the effects of forest patch size on H/L ratios were strongest in the enlarged broods, whereas the adverse patch size effect was not seen in the reduced broods. The brood H/L ratios were negatively associated with wing length, the amount of subcutaneous fat and pectoral muscle, body mass and haematocrit. Furthermore, high H/L ratios were related to increased nestling mortality suggesting that H/L ratios reflect nutritionally based physiological stress in bird young as recently suggested by Moreno *et al.* (2002). The H/L ratios are known to be sensitive to increased plasma

Table 1. Heterophil-lymphocyte (H/L) ratio and PHA response in the treecreeper broods (on day 11) in relation to the brood size manipulation (enlarged, control, reduced) and forest patch area. (Abbreviations used: (-), direction of the effect.)

dependent variable	independent variables	χ^2	d.f.	<i>p</i>
H/L ratio (<i>n</i> = 60 broods)	brood size manipulation	18.08	2	0.0001
	forest patch size (ha)	(-) 8.36	1	0.0038
	interaction	9.56	2	0.0084
PHA response (<i>n</i> = 55 broods)	brood size manipulation	1.21	2	0.5469
	forest patch size (ha)	0.22	1	0.6419
	interaction	1.96	2	0.3744

Table 2. Correlations between H/L ratio, CMI and six condition indices in the broods of the treecreeper on day 11. Pearson correlation coefficients with *p*-values and sample sizes are presented.

	wing	fat	muscle	mass	haematocrit	CMI	H/L ratio
H/L ratio							
<i>r</i>	-0.368	-0.516	-0.543	-0.606	-0.434	-0.168	1.0
<i>p</i>	0.004	< 0.0001	< 0.0001	< 0.0001	0.001	0.216	—
<i>n</i>	60	60	60	60	59	56	61
CMI							
<i>r</i>	0.316	-0.125	0.149	0.290	-0.049	1.0	-0.168
<i>p</i>	0.0175	0.3570	0.2740	0.0301	0.7265	—	0.216
<i>n</i>	56	56	56	56	54	56	56

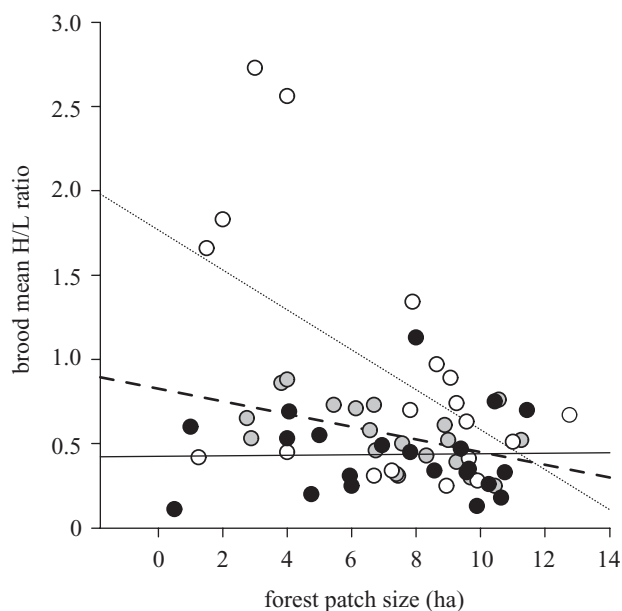


Figure 1. Brood mean H/L ratio in relation to forest patch area in the three brood manipulation groups. Enlarged broods (open circles, dotted line) ($\chi^2_{\text{GENMOD}} = 6.51$, d.f. = 1, $p = 0.0107$), control broods (grey circles, dashed line) ($\chi^2 = 5.93$, d.f. = 1, $p = 0.0149$), and reduced broods (black circles, solid line) ($\chi^2 = 0.01$, d.f. = 1, $p = 0.9215$).

corticosterone, which mediates nutritional stress (Morici *et al.* 1997; Post *et al.* 2003) and is known to promote severe protein loss, disrupt secondary messenger systems and suppress growth and metamorphosis when chronically elevated (Wingfield *et al.* 1997). Recent studies have also revealed effects of corticosterone-mediated nutritional stress on neuronal cell development and function in

growing chicks (Buchanan 2000; Buchanan *et al.* 2003), all of which may decrease the nestling and post-fledging survival.

The experimental evidence from a number of altricial passerines clearly demonstrates that the young are food limited because parents are limited in the quantity of food that they can supply relative to the energy demands of nestlings (Martin 1987). In forest-interior passerines, the loss of forest area may lead to chronic food shortage and consequently lowered breeding performance (Burke & Nol 1998; Zanette *et al.* 2000). The treecreeper is explicitly dependent on forested areas since it forages for invertebrates (primarily spiders) on tree trunks, the number of which may be lower in small forest fragments than in large fragments. In addition to the lower number of food patches (trunks), the invertebrate densities may be adversely affected by edge effects especially in small forest patches characterized by a high edge-area ratio. For example, surface-dwelling invertebrates are prone to desiccation, which may decrease their survival in edge habitat, characterized by a warmer and drier climate, as compared to that in forest interiors (Greenslade 1964; Matlack 1993). In our study area the parents forage up to a radius of 200 m from the nest (H. Hakkarainen, unpublished data). Hence, parents nesting in small forest patches may be forced to use neighbouring fragments, which in turn may lead to a lowered amount of food being delivered to the nestlings because of extended feeding trips and lowered feeding frequency.

Insectivorous passerines are especially susceptible to changes in weather conditions (Martin 1987). In our study area, the first broods of treecreepers are reared in variable weather conditions in spring, when the food resources on trunks are scant (Suorsa *et al.* 2003). In

changing environmental conditions, the young may store lipids and proteins to insure against the risk of food shortage (Martin 1987). We found that, in the small forest patches, nestlings had a lower body mass and lower subcutaneous fat and pectoral muscle condition suggesting that the food supply was primarily used for growth and maintenance, whereas in the large forest patches chicks were able to store fat and proteins as an insurance against temporary food shortage. In contrast to those of nestlings, the H/L levels and body condition of adults did not respond to the brood manipulation experiment or to the size of a forest patch. This suggests that parents did not increase parental effort with increasing within-brood competition. As a result, the costs of low territory quality, i.e. small forest patch size and elevated within-brood competition, were explicitly seen in nestlings.

There was no effect of our brood size manipulation or forest patch size on the cell-mediated response to PHA injection. This may have resulted from the fact that T-cell-mediated immunity does not react to the nutritional stresses in young treecreepers. Alternatively, the PHA method used may not have been appropriate for detecting nutritionally based stress responses in young treecreepers. In short, our experimental results emphasize that one reason for the area sensitivity in forest-interior species may be food shortage in small habitat patches with a potential to induce nutritional stress in growing young and lower the quality and survival of offspring.

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